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Assessment of Heavy Metals Tolerance in Leaves, Stems and Flowers of *Stevia rebaudiana* Plant

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Abstract

Stevia has become rather widespread over a wide range of climatic locations around the world and can apparently be successfully grown under different cultivation conditions. Heavy metal accumulation of Stevia extract is dependent on obtaining heavy metals from the soil and water. Heavy metals from plant sources may also vary from place to place because soil heavy metals content varies geographically, thus, they have become the subject of many research projects. Stevia plant is easily contaminated during growth, development and processing and for this, an extensive research is needed to explore the characteristics of the heavy metal produced by the plant. The heavy metals produced from the herb and its toxicity of Stevia plant is not well documented and scientific evidence is limited to establishing Stevia plant as a medicinal plant. The samples were collected from Malacca, Malaysia. The fresh leaves, stems and flowers of the *Stevia rebaudiana* plant were dried using oven equipment and were grinded until fine to make powder and then of each extracted using Microwave digester. The analysis of samples was carried out by using an Inductively Coupled Plasma Mass-Spectrophotometer (ICP-MS) with different mode equipment to compare results of heavy metals in *Stevia rebaudiana* plant. Heavy metal accumulation in *Stevia rebaudiana* from leaves, stems, and flowers extraction is reported. Heavy metals in leaves, stems and flowers of *Stevia rebaudiana* presented variety of elements such as As, Cd, Cr, Cu, Fe, Mg, Pb, Se, Zn, Al, Ag, Co, Ca, Mn and Ni. The high tolerance to heavy metals in leaves, stems, and flowers of *Stevia rebaudiana* were presented at fifteen parameters below the permissible limit in plant and can be used as food product or therapeutic agent in traditional medicine.

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1. Introduction

Recently, there has been an upsurge of interest in the use of substances of natural origin especially herbs of plants for therapeutic purposes such as the plant of Stevia which has been used as an herbal sweetener for years. However, the environmental conditions of the region and the agricultural practices decide about the level of heavy metals that could accumulate in the plant of Stevia used as herbal raw materials and also often used as food, functional food, nutritional or dietary supplements.

Over the last decades, environmental contamination with heavy metals has increased drastically [1]. Soil pollution with heavy metals will lead to losses in agricultural yield and hazardous health effects as they enter into the food chain [2]. It has been observed that agricultural soils have been contaminated due to he use of chemical fertilizers, pesticides, irrigation water and disposal of chemicals nearby. It is clear that quality control of herbal materials and medicinal plants is very important, not only for the safety of the herbal medicines themselves, but also for food safety. It should be borne in mind that although controlling the contaminants in plant of the herb has certain similarities, there may also be many differences. Therefore, more research is needed in order to establish the scientific evidence criteria for herbal medicines.

Many species of plants have been successful in absorbing contaminants such as lead, cadmium, chromium, arsenic, and various radionuclides from soils. One of phytoremediation categories, phytoextraction, usually can be used to remove heavy metals from soil using its ability to uptake metals which are essential for plant growth (Fe, Mn, Zn, Cu, Mg, Mo, and Ni). Some metals with unknown biological function (Cd, Cr, Pb, Co, Ag, Se, and Hg) can also be accumulated [3].

The bioavailability of metals in soil is a dynamic process that depends on specific combinations of chemical, biological, and environmental parameters [4-6]. In heavy metal polluted soils, plant growth can be inhibited by metal absorption. However, some plant species are able to accumulate fairly large amounts of heavy metals without showing stress, which represents a potential risk to animals and humans [7]. The level of plant tolerance to heavy metals is related to the balance between the rate at which metal ions are taken up and the efficiency with which they are detoxified within the plant. Thus the same amount of a metal present in plant tissues may be detrimental for one species while not at all for others [8-10].

Stevia plant is of worldwide importance today because its leaves are used as non-nutritive high potency sweetener primarily in Japan, Korea, China and South America. The consumption of Stevia extract in Japan and Korea was about 200 and 115 tons/year, respectively [11]. Stevia extracts having up to 300 times the sweetness of sugar, has garnered attention with the rise in demand for low-carbohydrate and low-sugar food alternatives. Because Stevia has a negligible effect on blood glucose, it is attractive as a natural sweetener for people on carbohydrate-controlled diets.

Stevia has become rather widespread over a wide range of climatic location around the world and can apparently be successfully grown under different cultivation condition, although often by seedling establishment in a greenhouse before planting in the field. Stevia plant is easily contaminated during growth, development and processing, and for this, an extensive research is needed to explore the characteristics of the heavy metal produced by a plant of Stevia. The heavy metals produced from the herb and its toxicity of Stevia plant is not well documented and scientific evidence is limited to establishing Stevia plant as a medicinal plant. Although there is a great concern about heavy metal contamination of herbal raw materials, information from the World Health Organization (WHO) regarding permissible limit is available only for Pb and Cd. Heavy metal accumulation of the *Stevia rebaudiana* extract is dependent on an obtained heavy metals from the soil and water. Heavy metals from plant sources may be also vary from place to place because soil heavy metals content varies geographically, thus, they have become the subject of many research projects.

2. Materials and methods

2.1. Extraction for mineral analysis

Three sections of the *Stevia rebaudiana* plant were used for the experiment. The section consists of leaves, stems and flowers. The raw material was purchased from Malacca, Malaysia. The materials were dried using oven equipment at 60°C for 24 hours to remove the moisture contains in leaves, stems and flowers. The dried materials were grinded to produce a powder and were measured the particle size (160µm) using a vibrator sieve shaker to give a good surface area for the extraction process. For the heavy metals determination, leaves, stems, and flowers of *Stevia rebaudiana* plants of each weighed approximately 0.5 grams into a Teflon digestion vessel, to which was added exactly 7 ml high purity concentrated 65% of nitric acid (HNO_3) and 1 ml high purity concentrated 30% of hydrogen peroxide (H_2O_2). The vessels were then capped and fitted into rotor segments and inserted into the microwave digestion system. The samples were radiated for 20 min. Upon cooling, the vessels were uncapped and solutions transferred into 50 ml volumetric flasks.

2.2. Analysis of heavy metals using ICP-MS

The analysis of samples (after extracted using microwave digestion system) was carried out by using an Inductively Coupled Plasma Mass-Spectrophotometer (ICP-MS) instrument Elan 9000 and Agilent 7500a to analyze levels of various heavy metals (As, Cd, Cr, Cu, Fe, Mg, Pb, Se, Zn, Al, Ag, Co, Ca, Mn and Ni). Five point calibration curves were constructed using five concentrations (0-500µg/L) for standards. 2% of nitric acid (HNO_3) was used as a blank. The sample introduced into the ICP-MS in a liquid form, was pumped into the sample introduction system, which was made up of a spray chamber and a nebulizer. The plasma (a highly ionized gas) contains different heating zones where the sample was successively dried, vaporized, atomized, and ionized. During this time, the sample was transformed from a liquid aerosol of solid particles. Then, into a gas contains the highest population of excited atoms and monotonic positively charged ions, representing the elemental composition of the sample. The highly efficient ion extraction, ion transportation through the mass spectrometer and detection was what gives to ICP-MS its ultratrace elemental detection features.

3. Results and discussion

Table 1. Total concentration of heavy metals normal range in plants, normal range in soils and toxic soils for plants.

Heavy metals	Normal range in plants (mg/kg^{-1})	Ref.	Normal range in soils[a] (mg/kg^{-1})	Toxic soils for plants[b] (mg/kg^{-1})
Arsenic (As)	5	[13]	6	20
Cadmium (Cd)	2	[14]	0.35	3 – 8
Chromium (Cr)	0.006 – 18	[15]	70	75 – 100
Copper (Cu)	0.4 – 45.8	[16]	30	60 – 125
Iron (Fe)	640 – 2486	[17]	–	–
Magnesium (Mg)	0.73 – 1.41	[18]	–	–
Plumbum (Pb)	3	[19]	35	100 – 400
Selenium (Se)	0.002 – 0.08	[20]	–	–
Zinc (Zn)	1 – 160	[16]	90	70 – 400
Aluminium (Al)	200 – ≥1000	[21-23]	–	–
Silver (Ag)	0.01	[24]	–	–
Cobalt (Co)	0.1 – 10	[25]	8	25 – 50
Calcium (Ca)	1830.2 – 2042.5	[26]	–	–
Manganese (Mn)	15 – 100	[27]	1000	1500 – 3000
Nickel (Ni)	0.1 – 3.7	[16]	50	100

a [28], b [29-30]

Plants are a good source for bioaccumulation of heavy metals. The influence of environmental factors and the type of plant itself, the levels of heavy metals in plants (both terrestrial and aquatic) vary widely [12]. Because the plants can reveal tolerance to contaminants. Thus, the concentration of essential and non-essential heavy metals in medicinal plants beyond permissible limit is a matter of great concern to public safety all over the world.

An assessment of heavy metals tolerance should be based upon a comprehensive analysis of the interaction between the accumulation of heavy metals in plants and the metal's status in soils. By specific review several of the total concentration permissible limits of heavy metals in plants and soils can be demonstrated for Stevia plant to the comparison as the control and the results can be assessed as heavy metals tolerance in the plant of *Stevia rebaudiana*. The total concentration of heavy metals normal range in plants, normal range in soils and toxic soils for plants listed in Table 1 are for to compare and information only. If the other facts are constants, the uptake of a metal by different plant species may be compared.

Inductively coupled plasma mass spectrophotometry (ICP-MS) has become a popular technique in the multi element analysis since the first commercial instrument became available in 1980s. Semi quantitative analysis by ICP-MS has proven to be a powerful tool for fast screening, in addition, it does not require the element of interest to be present in the calibration standard [31,32], making it especially useful for the analysis of unknown samples.

In this study, the analysis of samples was carried out by using an Inductively Coupled Plasma Mass-Spectrophotometer (ICP-MS) after extracted using microwave digestion system. The concentration of heavy metals determined with different mode equipment to compare results of heavy metals in *Stevia rebaudiana* plant. The analysis was carried out by using ICP-MS instrument Elan 9000 to analyze fifteen heavy metals were measured such as As, Cd, Cr, Cu, Fe, Mg, Pb, Se, Zn, Al, Ag, Co, Ca, Mn and Ni in leaves, stems and flowers of the *Stevia rebaudiana* plant. Meanwhile, analysis was carried out by using ICP-MS instrument Agilent 7500a to analyze nine heavy metals were measured such as As, Cd, Cr, Cu, Fe, Mg, Pb, Se and Zn in leaves, stems and flowers of the *Stevia rebaudiana* plant as shown in Table 2.

Table 2. The accumulation of heavy metals in Stevia rebaudiana (leaves, stems and flowers) by using ICP-MS Elan 9000 and Agilent 7500a.

Instruments	Elan 9000			Agilent 7500a		
Heavy metals	Leaves (mg/kg^{-1})	Stems (mg/kg^{-1})	Flowers (mg/kg^{-1})	Leaves (mg/kg^{-1})	Stems (mg/kg^{-1})	Flowers (mg/kg^{-1})
Arsenic (As)	0.05316	0.438	0.1728	0.0895	3.909	0.3135
Cadmium (Cd)	0.3515	0.361	0.169	0.6048	0.3571	0.2266
Chromium (Cr)	5.0103	3.87	4.01	5.621	6.1815	5.127
Copper (Cu)	6.9411	8.11	2.004	3.402	5.6917	6.461
Iron (Fe)	623	472	972	719	125.11	614
Magnesium (Mg)	189.56	1760	481.2	1.701	1034.2	1.76
Plumbum (Pb)	0.5233	0.513	1.1	1.682	0.4895	1.735
Selenium (Se)	0.00492	0.04536	0.03288	0.0007644	0.0246	0.0007644
Zinc (Zn)	44.4235	27.9	36.9	33.03	20.19	31.46
Aluminium (Al)	58.87	108.84	494.4	ND	ND	ND
Silver (Ag)	0.0676	0.0317	0.035	ND	ND	ND
Cobalt (Co)	0.0842	0.0611	0.0228	ND	ND	ND
Calcium (Ca)	259.44	558	450	ND	ND	ND
Manganese (Mn)	6.0358	6.336	12.48	ND	ND	ND
Nickel (Ni)	1.6737	1.53	2.4	ND	ND	ND

ND = No determined

The maximum values for heavy metals in herbal drugs and extracts have been discussed by several authors. In 1998, Kabelitz [33] published a detailed evaluation of a database on heavy metals, which included more than 12 000 samples originating from quality control analyses by several pharmaceutical companies. On this basis, maximum levels of Pb of 10 mg/kg^{-1} and for Cd of 0.5 mg/kg^{-1} were proposed. In this context, the WHO again proposes a limit of 10 mg/kg^{-1} for plumbum and 0.3 mg/kg^{-1} for cadmium dried herbs [34]. The concentrations of plumbum and cadmium in leaves, stems and flowers of *Stevia rebaudiana* given in Table 2, assessed are in the range that classifies them as a low source of plumbum and cadmium according to Kabelitz and WHO propose.

Arsenic can be hazardous for humans and animals when ingested as fine particles of dust or consumed in contaminated water. The uptake of arsenic by plants is associated with a change in speciation (methylated or complexes as phytochelatins) that also changes its toxicity, although some authors consider that plants rarely accumulate Arsenic to toxic levels [35]. If viewed concentration of arsenic in leaves, stems and flowers of *Stevia rebaudiana* as shown in Table 2, these were still lower than the total concentration normal range arsenic in plants, see Table 1.

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Chromium is a non-essential and toxic metal to plant growth, and it may be possible that plants do not have any specific mechanism of transport of Cr [36]. Chromium compounds are highly toxic to plants and are detrimental to their growth and development. Although some crops are not affected by low Cr concentration ($3.8x10^{-4}$ μM) [37, 38], Cr is toxic to higher plants at 100 μM. kg^{-1} dry weight [39].

The uptake of the copper from the soil by plants depends on the ability of the plants to transfer the metal across the soil–root interface and the total amount of copper present in the soil [40]. The normal heavy metal contents of terrestrial plants growing in uncontaminated soils were found to be in the range 0.4 to 45.8 mg kg^{-1} for Cu [16]. Copper (Cu) is necessary for plant growth in low concentrations, a structural part of enzymes, and is taken up as the divalent cation (Cu^{2+}) or Cu chalet. Copper, however, is often present in high concentrations that are toxic enough to biota. If viewed concentration of chromium and copper in leaves, stems and flowers of the *Stevia rebaudiana* plant as shown in Table 2, these were still in normal range.

Iron is essential for plant growth and is generally considered to be a micronutrient. Iron is considered the key metal in energy transformations needed for syntheses and other life processes of the cells [41]. Although iron itself is not considered toxic, it is environmentally significant because of its interaction with metals that are toxic. Iron oxides adsorb many elements and participate in the attenuation of most trace and heavy metals. Excess amounts of heavy metals, and of manganese, nickel, and cobalt in particular, caused a reduction in absorption and translocation of iron and resulted in a decrease of chlorophyll. On the other hand, high levels of iron compounds in the soil are known to greatly decrease trace metal uptake. Reactions between iron and manganese are commonly observed and the ratio of these two metals in both growth medium and plant tissue seems to be more important to plant metabolism than their concentrations [42, 43]. The result shows in Table 2, that by using Elan 9000, iron in leaves *Stevia rebaudiana* was 623 mg/kg^{-1}. Meanwhile, the result of iron in stems was 472 mg/kg^{-1} and in flowers was 972 mg/kg^{-1}. Then, the result shows in Table 2 that by using Agilent 7500a, iron in leaves of *Stevia rebaudiana* was 719 mg/kg^{-1}. Meanwhile, the result of iron in stems was 125.11 mg/kg^{-1} and in flowers was 614 mg/kg^{-1}.

The magnesium concentration of tissues considered as deficient, sufficient, or toxic depends on what growth parameter is being measured in the crops. In many food crops, classification of nutrient sufficiency is based on harvestable yields and quality of the edible plant parts [44]. According to Marschner [45], 15 to 30% of the total magnesium in plants is associated with chlorophyll molecule. Magnesium is an essential element in biological systems. Magnesium occurs typically as the Mg^{2+} ion. It is an essential mineral nutrient for life. The result shows in Table 2 that by using Elan 9000, magnesium in leaves *Stevia rebaudiana* was 189.56 mg/kg^{-1}. Meanwhile, the result of magnesium in stems was 1760 mg/kg^{-1} and in flowers was 481.2 mg/kg^{-1}. Then, the result as shown in Table 2 by using Agilent 7500a, magnesium in leaves of *Stevia rebaudiana* was 1.701 mg/kg^{-1}. Meanwhile, the result of magnesium in stems was 1034.2 mg/kg^{-1} and in flowers was 1.76 mg/kg^{-1}.

Some plants are tolerant to selenium, showing a high capability to accumulate this element without symptoms of toxicity. They are also capable of translocating and/or transforming selenium into bioactive compounds, which play an important role in both, human nutrition and phytoremediation [46]. The results of selenium in leaves, stems, and flowers of the *Stevia rebaudiana* plant were extremely lower, as shown in Table 2.

Zinc is an essential element for plant nutrition. It plays structural and/or catalytic roles in many enzymes such as Cu–Zn superoxide dismutase, alcohol dehydrogenase, RNA polymerase and DNA-binding proteins and is associated with the carbohydrate metabolism [47,48]. The normal heavy metal contents of terrestrial plants growing in uncontaminated soils were found to be in the range of 1–160 mg kg^{-1} for Zn [16].

Generally, for most plant species and especially crop species, aluminium tolerance is interpreted as the ability to exclude aluminium. The exudation of organic acids from the roots is considered one of the most important strategies by which aluminium is excluded [49]. Aluminium accumulator species are distributed in acid soils, particularly in the tropics. More than half a century ago, Chenery [22,23] determined the aluminium concentrations in the leaves of thousands of plant species and classified them as aluminium accumulators ($\geq$1000 mg Al kg^{-1}) or Al non-accumulators (<1000 mg Al kg^{-1}). He reported that 1779 of 2859 species in dicots, 33 of 269 species in monocots and gymnosperms, and 615 of 1401 species in cryptogams were aluminium accumulators. The accumulation of aluminium in leaves, stems, and flowers of *Stevia rebaudiana* shown in Table 2 were an aluminium non-accumulator.

Silver is a non-essential heavy metal for any living organism, which enters the aquatic environment from natural and anthropogenic sources (photographic processing effluents, sewage sludge, biocidal and other applications). It is known that Ag ions interact metabolically with Cu and Se and replace H_2 from the sulfhydryl groups of the photosynthetic enzymes (such as Rubisco), changing their structure and inactivating them. Ag also forms complexes with amino acids, pyrimidines, purines and nucleotides, as well as with their corresponding macromolecular forms, suggesting its potential to be either highly toxic or easily inactivated by the plant [50]. However, there are very few toxicological data of silver in plants. Silver content in plant tissue is usually less than 0.01 mg kg^{-1} [24]. The result shows in Table 2 that silver in leaves of *Stevia rebaudiana* was 0.0676 mg/kg^{-1}. Meanwhile, the result of silver in stems was 0.0317 mg/kg^{-1} and in flowers was 0.035 mg/kg^{-1}.

While it has been known for many years that Cobalt is an essential element for humans, animals and prokaryotes, a physiological function of this element in higher plants has not been identified. The Co-containing vitamin B12 does not occur in plants. Whereas normal Cobalt concentrations in plants are cited to be as low as 0.1–10 mg kg^{-1} dry weight, its beneficial role as a trace element has been described [25]. Calcium is an essential plant nutrient, whose role has been well documented [51]. Calcium has an important role in plant physiology, including involvement in the responses to stress, and controls numerous processes [52]. The result shows in Table 2 that calcium in leaves of *Stevia rebaudiana* was 259.44 mg/kg^{-1}. Meanwhile, the result of calcium in stems was 558 mg/kg^{-1}and in flowers was 450 mg/kg^{-1}.

Manganese is an essential element in plant growth, but excessively high levels of Mn in soil can also hamper plant growth tremendously [53]. The result as shows in Table 2 that manganese in leaves of *Stevia rebaudiana* was 6.0358 mg/kg^{-1}. Meanwhile, the result of manganese in stems was 6.336 mg/kg^{-1} and in flowers was 12.48 mg/kg^{-1}.

Nickel is also a non-essential metal like Cr but it may be attributed that hyper accumulators of Ni metal can store it into vacuoles of leaves to protect the plants from Ni toxicity [54]. The normal heavy metal contents of terrestrial plants growing in uncontaminated soils were found to be in the range 0.1 to 3.7 mg kg^{-1} for Ni [16]. The result shows in Table 2 that nickel in leaves of *Stevia rebaudiana* was 1.6737 mg/kg^{-1}. Meanwhile, the result of nickel in stems was 1.53 mg/kg^{-1} and in flowers was 2.4 mg/kg^{-1}.

In general, the results have shown that accumulation of heavy metals in leaves, stems and flowers of *Stevia rebaudiana* carried out by using ICP-MS instruments Elan 9000 and Agilent 7500a did not reach phytotoxic concentrations or toxic levels, see Table 1 for reference values. Thus, the results have shown that they can be used as current information on the actual situation regarding of heavy metals in plants of Stevia used as herbal raw material for medicinal or food purposes. In this context, a proposals for maximum levels for heavy metals in herbal drugs have been documented to support activities control metal contamination in herbal medicinal products and food supplements.

4. Conclusion

Nowadays, a high degree of contamination by heavy metals can be observed in the environment. The plant of *Stevia rebaudiana* which is used as an herbal sweetener can be contaminated directly of heavy metals during growth by soils and water. At the same time, a plant is a member of the food chain and may create a risk for human and other animals through contamination of food supplies. The tolerance to heavy metals in leaves, stems, and flowers of the *Stevia rebaudiana* plant were presented a variety of elements such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), plumbum (Pb), selenium (Se), zinc (Zn), aluminium (Al), silver (Ag), Cobalt (Co), calcium (Ca), manganese (Mn) and nickel (Ni). In general, the determined concentration of heavy metals in leaves, stems and flowers of *Stevia rebaudiana* were below the tolerance level. The implication of the findings may be taken into consideration whilst using the herbs for food safety in preparation of herbal products and standardized extracts should be collected from an unpolluted natural.

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